# Structural and dynamical characterization and binding investigation of Calpastatin by NMR spectroscopy

Ph.D. theses

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## Introduction

Calpain is an intracellular calcium–activated cysteine protease present in all eukaryotic cells. It controls the activity of over one hundred target proteins by limited proteolysis in the regulation of cell division, differentiation and cell motility. It occurs together with its endogenous protein inhibitor, calpastatin. Calpastatin is an intrinsically disordered protein, composed of an N-terminal L-domain and four equivalent inhibitory domains, each of about 140 amino acids in length Every inhibitory domain contains three short conserved segments, termed subdomains, which are primarily responsible for calpastatin binding to calpain. Subdomain A and C potentiate inhibition, as they tether the inhibitor to the enzyme in a calcium–dependent manner, but do not inhibit enzyme activity on their own. The middle segment, B, binds in the active–site region of the enzyme and carries inhibitory potential in itself.

In its unbound form, calpastatin lacks a well-defined structure and falls into the class of intrinsically disordered proteins, IDPs. IDPs play important roles in signal transduction and transcription regulation. Uncovering their

bound states and the process of recognition is key to understand IDP action at the molecular level, as well as to extend the structure-function paradigm.

The uniqueness of the interaction of calpastatin with calpain stems from biochemical data also suggesting its tripartite binding, with the involvement of the three subdomains and the intervening disordered linker regions. Due to technical difficulties, however, the structure of the calpain-calpastatin complex has not yet been solved.

**Aims**

Our first goal was to carry out the total NMR assignment of three different form of calpastatin (hCSD1, hCSD1_$Ca^{2+}$, hCSD1$^{(67-141)}$). We aimed to investigate the dynamical and structural features of hCSD1 in its free form highlighting the secondary structural elements. Moreover we wished to use NMR-spectroscopy to observe the binding process between calpain and calpastatin.

**Methods**

*Protein expression and purification:* The $^{15}N$ and $^{15}N$-$^{13}C$-labeled calpastatin variants as well as $C^{105}S$ inactive calpain mutant were produced by using *E.coli*. strain. Prior to

NMR measurements the samples were purified on C-18 column using HPLC system.

*NMR resonance assignment:* The resonance assignment of backbone carbon and nitrogen atoms were obtained from HNCA / HN(CO)CA, HNCO / HN(CA)CO and HNCACB / HN(CO)CACB experiments, while the proton assignment were accomplished using 3D {$^{1}$H, $^{15}$N, $^{1}$H}-HSQC-TOCSY and 3D {$^{1}$H, $^{15}$N, $^{1}$H}-HSQC-NOESY spectra.

*Spin-labelling:* A functional group that contain unpaired electron was attached to the $C^{103}$ residue of hCSD1, and its effect on relaxation of residues were monitored by lineshape analysis of $^{15}$N-$^{1}$H-HSQC peaks. By this method the long-range intramolecular interactions were investigated.

*Reduced spectral density mapping:* Relaxation data were analyzed using the reduced *spectral density mapping* approach. Spectral density at given frequencies were calculated form $T_1$, $T_2$ and heteronuclear-NOE of the amide nitrogen atoms.

*Single motion curve analysis*: Characteristic correlation times of hCSD1 and possible chemical exchange processes were analyzed by *single motion curve* method.

*Analysis of binding to calpain:* Calpain titration was performed by adding small amounts of calpain to $^{15}$N-labelled hCSD1. During titration steps with calpain HSQC spectra were collected in each step and peak intensities were used to characterize binding to calpain.

**Results**

*1. The total NMR assignment of first functional domain of human calpastatin (hCSD1) and its 74 residue long C-terminal region (hCSD1$^{(67-141)}$) were carried out.* $^{15}$N- and $^{15}$N- $^{13}$C-labelled form of first functional domain of human calpastatin (hCSD1) and its 74 residue long C-terminal region (hCSD1$^{(67-141)}$) were expressed in *E.coli*. Based on $^{1}$H-$^{15}$N HSQC, 3D {$^{1}$H, $^{15}$N, $^{1}$H}-HSQC-TOCSY and 3D {$^{1}$H, $^{15}$N, $^{1}$H}–HSQC-NOESY as well as HNCA / HN(CO)CA, HNCACB / HN(CO)CACB and HNCO / HN(CA)CO triple resonance NMR measurements the total assignments of calpastatin forms were carried out. In case of hCSD1 122 out of 126 non-prolin peaks were identified in $^{1}$H-$^{15}$N HSQC spectrum, while 63 out of 66 non-prolin peaks in spectrum of a hCSD1$^{(67-141)}$ were assigned.

*2. pH and temperature dependence of spectra of hCSD1 were analysed.* Changes in lineshape and positions of peaks in $^{1}H$-$^{15}N$ HSQC spectra of hCSD1 were monitored while pH and temperature were shifted. We found that the optimal values of pH and temperature are pH~6 and 298 K for NMR measurements.

*3. Analysis of secondary chemical shift values of hCSD1 and hCSD1$^{(67-141)}$ reveals subdomain B to be partially ordered.* By comparison of chemical shifts values of hCSD1 and hCSD1$^{(67-141)}$ we showed that the subdomain B has some transient structural elements which motifs are disappeared by truncation in TIPPKYR segment.

*4. We found that the calpastatin able to bind $Ca^{2+}$-ion and this process has influence on structural properties of the molecule.* Total NMR assignments of calpastatin were performed at different $Ca^{2+}$-ion concentrations. By comparison of chemical shift values it became obvious that the Glu-rich regions of calpastatin are able to bind calcium ion. Moreover by addition of $Ca^{2+}$-ion the transient structural motifs can be stabilized due to the compensation the negative charges of side chains of glutamic acid.

*5. The spin-labeling measurements reveal the lack of*

*long-range interactions in hCSD1.* A functional group that contain unpaired electron was attached to the $C^{103}$ residue of hCSD1. Residues in ~25Å area from functional group suffer decrease in signal intensity of HSQC peaks. Almost each residue was influenced by effect of unpaired electron which suggest that the role of the long range intramolecular interactions can be neglected.

6. *Analysis of secondary chemical shift values and dynamical parameters reveals the presence of transient secondary structural elements.* The analysis of secondary chemical shift values of free form of hCSD1 reveals some helical structural character at functional subdomains. The dynamical parameters in these regions also show decreased flexibility. Moreover the N- and C-terminal regions as well as residues between functional subdomains exhibit greater structural mobility. Based on both dynamical and structural properties a new "subdomain" was defined and this finding was supported by *sequential alignment* results.

*7. The binding process of calpain-calpastatin was investigated by NMR.* Binding process of calpain–calpastatin was monitored through the changes of chemical shift values and relaxation properties of $^{15}$N-labelled calpastatin in

presence of calpain. It was proven that residues of sub-domain A and C bind to the calpastatin immediately while the binding of sub-domain B is only partial. The non-binding residues preserve their disorder and in spite of size of molecular assembly (123kDa) can be investigated by NMR-spectroscopy.

**Discussion**

Although the first domain of human calpastatin (hCSD1) is intrinsically unstructured our comprehensive NMR analysis point out some transient helical structures which are closely correspond to the functional subdomains. These structural features in free-state of the full length inhibitor, helps the extremely fast binding and inhibition of the enzyme, while preserve the advantages of disorder. Prior to our experiments it was known that the calpain bind calcium-ion to reach its binding conformation. Our analysis suggests that the calpastatin itself bind calcium as well and this interaction helps to stabilize the transient structural motifs.

Our results on calpain-calpastatin binding provide evidence for tripartite binding mode and confirm the previous

definitions of subdomains. Finally we have pointed out that in exploration of complexes of intrinsically unstructured proteins, NMR can be complementary method for X-ray crystallography.

## Corresponding publications

1. Kiss R., Kovacs D., Tompa P., Perczel A., (2008) Local structural preferences of calpastatin, the intrinsically unstructured protein inhibitor of calpain *Biochemistry* 47 (26) 6936–6945

2. Kiss R., Bozoky Z., Rona G., Friedrich P., Dvortsak P., Weisemann R., Tompa P., Perczel A., (2008) Calcium-induced tripartite binding of intrinsically disordered calpastatin to its cognate enzyme, calpain *FEBS Lett.* 582 2149–2154